## Inhibition of Methanogenesis in Marine Sediments by Acetylene and Ethylene: Validity of the Acetylene Reduction Assay for Anaerobic Microcosms

## RONALD S. OREMLAND AND BARRIE F. TAYLOR\*

Division of Biology and Living Resources, University of Miami, Rosenstiel School of Marine and Atmospheric Science, Miami, Florida 33149

Received for publication <sup>19</sup> May 1975

Methanogenesis was irreversibly inhibited in sediments by concentrations of acetylene employed in nitrogen fixation assays (1 to 20%, vol/vol). Ethylene, but not ethane, also stopped methane production, and the inhibition was reversed by gassing with hydrogen.

The acetylene reduction technique is widely used for field and laboratory estimations of nitrogenase activity (6). It was recently reported that some species of methane-oxidizing bacteria may be inhibited by acetylene and also oxidize ethylene (3), thereby complicating field assays (5). We now wish to draw attention to a further problem when the assay is used to measure nitrogen fixation in anaerobic microcosms, a problem due to the inhibition of methanogenesis by acetylene.

Sediments associated with communities of the sea-grass Thalassia testudinum possess methanogenic activity (R. S. Oremland and B. F. Taylor, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, N8, p. 185). The sediments from these marine angiosperm communities contained about 2% (dry weight) of organic carbon and 50 to 60% (by weight) water. Sediments were homogenized, in a Waring blender, with oxygen-free seawater. The homogenate was pipetted, in 20-ml portions, into 50-ml Erlenmeyer flasks, which were gassed with hydrogen or argon and then sealed with recessed butyl rubber stoppers. Oxygen was excluded during manipulations by the techniques described for the cultivation of strict anaerobes (7). Acetylene, ethylene, or ethane was injected to the desired concentration into the gas phase, and chloroform (0.05%, vol/vol), an inhibitor of methanogenic bacteria (1), was added to some flasks. The flasks were incubated at 26 C in the dark with reciprocal shaking. Hydrocarbons were determined, in the gas phase, by flame ionization chromatography with a stainlesssteel column (6 feet by 1/8 inch, outer diameter [ca. 182.9 by 0.38 cm]) containing porapak R (100 to 120 mesh) at 75 C with nitrogen as carrier gas (flow rate, 25 ml/min). Hydrogen was detected with a gas partitioner (thermal conductivity) using two aluminum columns in series containing, respectively, a molecular sieve (13 feet by  $\frac{3}{16}$  inch, outer diameter [ca. 396 by 0.5]  $cm)$ ) and hexamethyl-phosphoramide (6 feet by  $3/16$  inch, outer diameter). The columns were operated at 35 C with argon (60 ml/min) as carrier gas.

Methane production was inhibited by 1.25 to 20% (vol/vol) acetylene and did not resume when the flasks were gassed with hydrogen (Fig. 1). Lower concentrations of acetylene (0.13 and 0.01%) merely decreased the rate of methanogenesis. Ethylene was inhibitory at concentrations of 5 and 20%, but not at 1.25%, and the inhibition was relieved by subsequent gassing for 5 min with hydrogen at the lower but not the higher concentration (Fig. 2). The reversal of  $C_2H_4$  inhibition by gassing was presumably related to the extent of elimination of  $C_2H_4$ from the inhibited flasks, because gassing for 10 min with  $H_2$  reversed inhibition caused by  $20\%$  C<sub>2</sub>H<sub>4</sub>. Ethane (1.25 to 20%) had no effect on the rate of methane production. Methanogenesis in control flasks was unaffected by the injection of 0.5 ml of air either at zero time or during the exponential phase of methane production. The sediment homogenates were therefore presumably well buffered against oxygen, probably by sulfide and oxygen-consuming microbes, and the observed inhibitions were due to the unsaturated  $C_2$  hydrocarbons and not oxygen contamination. It is also relevant that unsaturated fatty acids inhibit methanogenesis (10).

A transient accumulation of hydrogen was observed in systems containing 20% acetylene in argon, whereas in sediments inhibited with both acetylene and CHCl<sub>3</sub> hydrogen accumulated and did not disappear (Table 1). The production of hydrogen in sediments inhibited with  $C_2H_2$  is similar to the effects observed with



FIG. 1. Irreversible inhibition of methanogenesis by 1.25% (vol/vol)  $C_2H_2$ . The Erlenmeyer flasks contained 20 ml of sediment homogenate (dry weight, about  $8g$ ) incubated at 26 C in the dark under hydrogen. Symbols: O, uninhibited control;  $\triangle$ , 1.25% C<sub>2</sub>H<sub>2</sub> ungassed;  $\triangle$ , 1.25% C<sub>2</sub>H<sub>2</sub> gassed at 30 h with H<sub>2</sub> (arrow);  $\Box$ , 125%  $C_2H_2$  and 0.05% (vol/vol) CHCl<sub>3</sub> gassed at 30 h with  $H_2$ . Gassing with  $H_2$  removed 99% of the  $C_2H_2$  from the gas phase.

 $chloromethanes$  (1), although CHCl<sub>3</sub> did not always cause hydrogen accumulation in our experiments. The eventual disappearance of hydrogen in the acetylene-inhibited homogenates is due to its consumption by other anaerobes (Oremland and Taylor, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, N8, p. 185) and possibly its reduction of acetylene. Ethane production was not detected in flasks containing non-inhibitory levels of ethylene, and at inhibitory concentrations the chromatographic peak for ethylene obscured that for ethane. However, systems inhibited with acetylene produced ethylene, and its rate of production was lower in flasks containing both acetylene and  $CHCl<sub>3</sub>(Ta$ ble 2). There appears to be a correlation between hydrogen consumption and ethylene production in the results shown in Tables <sup>1</sup> and 2, but the system is too complex to reach any firm conclusions. CHCl<sub>3</sub> may have retarded the reduction of acetylene by the methanogenic enzymatic systems, but it could also have inhibited a hydrogen-dependent nitrogenase activity of either methanogens or other anaerobes (e.g., clostridia containing  $B_{12}$  enzymes). Further work is needed to resolve this situation, particularly since it is still not known if methanogenic bacteria fix nitrogen (9). Pine and Barker (8) demonstrated  $^{15}N_2$  incorporation by Methanobacterium omelianskii, but subsequent investigations employing the acetylene reduction technique on the S organism and Methanobacte-



FIG. 2. Reversible inhibition of methanogenesis by 5% (vol/vol)  $C_2H_4$ . The Erlenmeyer flasks contained 20 ml of homogenized sediment (dry weight, about  $8g$ ) incubated at 26 C in the dark under hydrogen. Symbols:  $\bigcirc$ , uninhibited control  $\blacktriangle$ , 5%  $C_2H_4$ ungassed;  $\triangle$ , 5% C<sub>2</sub>H<sub>4</sub> gassed at 30 h with H<sub>2</sub> (arrow);  $\Box$ , 5%  $C_2H_4$  and 0.05% (vol/vol) CHCl<sub>3</sub> gassed at 30 h with  $H_2$ . Gassing with  $H_2$  removed 98% of the  $C<sub>2</sub>H<sub>4</sub>$  from the gas phase.

TABLE 1. Hydrogen concentrations in the gas phases of uninhibited and inhibited sediment systems<sup>a</sup>

	Hydrogen concn (nmol/ml) <sup>b</sup>			
Time (h)	No inhibitor	20% C.H.	$20\% \text{ C}_2\text{H}_2 +$ 0.05% CHCl <sub>3</sub>	
0	0			
5	26	26	18	
29	0	40	160	
52	0	17	166	
78			120	

<sup>a</sup> The Erlenmeyer flasks contained about 8 g (dry weight) of sediment and were sealed under argon.

 $b$  The limit for the detection of  $H<sub>2</sub>$  was about 6 nmol/ml.

TABLE 2. Methane and ethylene concentrations in the gas phases of uninhibited and inhibited sediment systems<sup>a</sup>

Time (h)	Methane concn (no inhibitor) $(nmol/ml)^b$	Ethylene concn $(nmol/ml)^c$	
		20% C.H.	$20\% \text{ C}_2\text{H}_2 +$ 0.05% CHCl,
0	0.80	0.30	0.36
6	0.86	0.54	0.46
21	2.62	1.22	0.76
29	5.38	1.09	0.86
43	9.34	1.97	0.98
68	10.34	3.17	0.90

<sup>a</sup> The Erlenmeyer flasks contained about 8 g (dry weight) of sediment and were sealed under argon.

<sup>b</sup> Ethylene was not produced in the absence of acetylene.

<sup>e</sup> Methane was not produced by inhibited systems.

rium MoH failed to specify which organism possesses nitrogenase (M. P. Bryant, personal communication), perhaps because acetylene inhibited the latter organism.

It is apparent, from the above results, that acetylene inhibits methanogenesis at concentrations used in the nitrogenase assay, but it is not clear if this causes an over- or underestimate of sediment nitrogen fixation rates. Hardy et al. (6) in their development of the acetylene reduction assay noted that acetylene markedly decreased methane formation by rumen contents and Elleway et al. (4) confirmed this observation. Neither group of workers, however, questioned the validity of the acetylene reduction assay for nitrogen fixation by anaerobic microbial communities. Even if methanogenic bacteria do not fix nitrogen, they probably play a symbiotic role in nitrogen fixation by anaerobic microbial communities, and, since Clostridium pasturianum is also adversely affected by acetylene (2), the acetylene reduction assay must be checked with the 15N method when studying anaerobic microcosms. Work is underway to further define the site and nature of the inhibition of methanogenesis by acetylene and ethylene.

## ADDENDUM

Since this communication was submitted for publication, Raimbault (11) reported the inhibition by acetylene of methane evolution from anaerobic paddy soils and the acetylene inhibition of methanogenesis and growth of a strain of Methanosarcina isolated from paddy soil.

We thank M. P. Bryant for his generous instruction in the techniques of anaerobic microbiology. We also thank C. C. Lee for data on the organic content of the sediments.

This research was supported by the Oceanography Section, National Science Foundation, NSF grant GA-41135.

## LITERATURE CITED

- 1. Bauchop, T. 1967. Inhibition of rumen methanogenesis by methane analogues. J. Bacteriol. 94:171-175.
- 2. Brouzes, R., and R. Knowles. 1971. Inhibition of growth of Clostridium pasteurianum by acetylene: implication for nitrogen fixation assay. Can. J. Microbiol. 17:1483-1489.
- 3. De Bont, J. A. M., and E. G. Mulder. 1974. Nitrogen fixation and co-oxidation of ethylene by a methaneutilizing bacterium. J. Gen. Microbiol. 83:113-121.
- 4. Elleway, R. F., J. R. Sabine, and D. J. D. Nicholas. 1971. Acetylene reduction by rumen microflora. Arch. Mikrobiol. 76:277-291.
- 5. Flett, R. J., J. W. M. Rudd, and R. D. Hamilton. 1975. Acetylene reduction assays for nitrogen fixation in freshwaters: a note of caution. Appl Microbiol. 29:580-583.
- 6. Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The acetylene-ethylene assay for  $N_2$ fixation: laboratory and field evaluation. Plant Physiol. 43:1185-1207.
- 7. Hungate, R. E. 1969. A roll-tube method for cultivation of strict anaerobes, p. 117-132. In J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press Inc., New York.
- 8. Pine, M. J., and H. A. Barker. 1954. Studies on the methane bacteria. XI. Fixation of atmospheric nitrogen by Methanobacterium omelianskii. J. Bacteriol. 68:589-591.
- 9. Postgate, J. R. 1974. Evolution within nitrogen-fixing systems, p. 263-292. In M. J. Carlile and J. J. Skehel (ed.), Evolution in the microbial world, Symp. Soc. Gen. Microbiol., vol. 24. Cambridge University Press, London.
- 10. Prins, R. A., C. J. Van Nevel, and D. I. Demeyer. 1972. Pure culture studies of inhibitors for methanogenic bacteria. Antonie van Leeuwenhoek J. Microbiol. Serol. 39:281-287.
- 11. Raimbault, M. 1975. Étude de l'influence inhibitrice de l'acétylène sur la formation biologique du méthane dans un sol de rizibre. Ann. Inst. Pasteur Paris 126A:247-258.